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INNATE IMMUNE RESPONSES TO MICROBIAL POISONS: Discovery and Function of the Toll-Like Receptors

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■ **Abstract** There are many circumstances under which a toxin exploits an endogenous receptor or another protein of host origin to work its untoward effects. In most instances, the receptor normally fulfills a function that has nothing to do with the toxin per se; that is, the toxin is not the "natural" ligand. The situation with endotoxin, however, is a remarkable one. The endotoxin receptor evolved to detect endotoxin. Why have mammals maintained a gene that can undermine their survival? The search for the endotoxin receptor answered this question and also revealed the essential function and biological strategy of the Toll-like receptors: principal sensors of the innate immune system.

INTRODUCTION

Vertebrates are endowed with two fundamental types of immunity. The more ancient system was discovered by Metchnikoff, close on the heels of the foundation of the germ theory of disease by Pasteur and Koch. Metchnikoff observed the engulfment of fungal spores by specialized cells of invertebrate organisms and correctly concluded that this must comprise an important means of host defense. Not long after, phagocytic cells were observed in mammals, including humans, and the general importance of phagocytosis was widely confirmed. Ehrlich, a contemporary of Metchnikoff, discovered antibodies in the blood of animals that had been infected or inoculated with deadly bacteria or bacterial toxins. Many years would elapse before it was realized that antibodies were produced by lymphocytes and before the cooperative nature of the adaptive and innate immune systems was well understood. In the fullness of time, it has become evident that adaptive immunity is, in many ways, subordinate to innate immunity. Without the vital antigen-presenting function of mononuclear phagocytic cells, including macrophages and dendritic cells, and without individual molecules of myeloid origin, adaptive immune responses cannot be initiated. Moreover, immunodeficiencies that involve defects of the

myeloid lineage are often more severe than those that involve defects of the lymphoid lineage. But for much of the twentieth century the identity of receptors utilized by the innate immune system remained a puzzle.

The discovery of the afferent (sensing) system used by innate immune cells to perceive infection has been a major advance in immunology. The roots of this discovery were firmly anchored in genetics, toxicology, and microbial pathogenesis. A focused investigation into the mechanism of endotoxin (lipopolysaccharide) toxicity ultimately led to the understanding that the Toll-like receptors (TLRs), ten of which are encoded in the human genome, are the essential sensors that activate both innate and adaptive immune responses.

In this review, the critical events and discoveries leading to the elucidation of TLR function are presented, along with inferences that have since been drawn concerning signal transduction, genetic diversity, and pharmacotherapeutic opportunities that have arisen from the study of this class of proteins.

HISTORY

As reviewed by Rietschel & Cavaillon (1–3), the term endotoxin was coined by Richard Pfeiffer, a student of Robert Koch. Pfeiffer first identified endotoxin as an agent responsible for fever and shock in animals that were injected with heat-killed preparations of *Vibrio cholerae*, or organisms that had been neutralized with antibodies (4). Until the end of his career, he was unaware that the O-antigens of gram-negative bacteria were covalently attached to the substance he had called endotoxin. It fell to Boivin, Staub, Lüderitz, and others to demonstrate the lipopolysaccharide nature of endotoxin, to show that it was the principal glycolipid component of the outer membrane of gram-negative bacteria, and ultimately, to solve its chemical structure (5–8).

Endotoxin then became a toxin in search of a mechanism. As often happens, the first steps to be taken in finding the mechanism were partly descriptive. A great deal of early work was directed toward understanding precisely what endotoxin did within the mammalian host. As Pfeiffer had observed, much of the toxicity of a gram-negative infection seemed ascribable to endotoxin itself. But perhaps not all of the toxicity can be so ascribed, and it is worth noting that Pfeiffer mistakenly remarked on the presence of "endotoxin" in certain gram-positive bacteria (1). This bespeaks the similarity between the biological effects of true endotoxin and other microbial substances, including peptidoglycan, lipopeptides, and unmethylated DNA.

Endotoxin (from this point forward used interchangeably with LPS: lipopolysaccharide) is, for the most part, poisonous only to mammals. In embryo, birds are also sensitive to LPS (9). But in late embryonic life, a high state of resistance is acquired. Adult birds are scarcely sensitive to LPS at all (10). Among mammals, a haphazard pattern of sensitivity is observed (10). Ungulates, rabbits, anthropoid

apes, and humans are all extremely sensitive to LPS, whereas lower primates, rats, and mice are quite resistant. In all mammalian species, infusion of LPS causes fever, and immediate neutropenia produced as a result of margination of neutrophils within the vascular tree (11–14). Many neutrophils marginate in the lungs, and some extravagate into the air spaces, producing acute respiratory distress (11). Endotoxin is also known to modify the anticoagulant surface of vascular endothelium, permitting the deposition of fibrin and actually depleting fibrin from the plasma (15, 16). A prompt fall in blood pressure, caused partly by vasodilatation and partly by diminished cardiac output, leads to hypoperfusion of vital organs and ischemic injury (15–17). Ischemia of the intestinal tract can lead to the influx of still more endotoxin, sealing the fate of the affected individual (18). Endotoxin also sets in motion long-term effects that are still ill understood: It may influence the character of adaptive immune responses that far outlast the innate immune response.

Many poisons ultimately create a complex picture of deranged physiology so that an investigator is hard pressed to assign culpability to an initial event. Hence, it was once suspected that LPS might intercalate into the membranes of cells, creating ionophoric effects or signaling in the absence of a receptor. It was also felt that LPS might interact with many receptors. Or it might have a primary effect on blood coagulation.

The power of a pure genetic approach to the question of mechanism was made clear in 1965, when it was observed that mice of the C3H HeJ strain were highly resistant to the biological effects of LPS (19). In time, it became apparent that the phenotype of these mice was quite specific for LPS; other bacterial toxins provoked a normal response in these animals. Mice of the C3H/HeJ strain sustained a mutation that became fixed in the population within the first years of the 1960s. They were hypersusceptible to authentic gram-negative infections (20, 21), and a single gene was shown to be involved in the phenotypic difference (22, 23). A second strain of mouse was also found to be LPS resistant and to have an allelic disorder (24, 25). In aggregate, two very important lessons were absorbed.

First, however complex the interaction between LPS and cells and proteins of the host, the product of a single gene was required for all of the toxicity of LPS. This observation spoke strongly in favor of a single protein receptor target for LPS.

Second, it began to be quite clear that LPS was “intentionally” sensed. In other words, sensing LPS was advantageous to the host. Infection with gram-negative bacteria could not be eliminated effectively in the absence of LPS sensing.

The stage was then set for the solution to two of the central questions in the LPS field: Why does the host maintain a system that can produce such fearsome injury? Why is it advantageous to sense LPS at all? The inescapable conclusion has been that LPS sensing is a phenomenon intended to deal with small inocula of gram-negative organisms. More detailed discussion is given to this notion below.

THE ROLE OF HEMATOPOIETIC CELLS IN ENDOTOXIN SENSING

Although endotoxin interacts with many cells throughout the body, and probably does intercalate into membranes in a fairly nonspecific fashion, the lethal effect of LPS seems to be conferred by cells of hematopoietic origin. This fact was revealed by adoptive transfer studies in which crosstransplantation of C3H/HeJ and C3H/HeN hematopoietic stem cells was carried out after lethal irradiation (26). The phenotype of the donor determined the phenotype of the radiation chimera. In separate experiments, it was shown that macrophages were of principal importance in LPS toxicity (27, 28). As hematopoietic derivatives, macrophages therefore seem to be the most important responder cells, ultimately conferring the lethal effect of LPS.

In 1985, tumor necrosis factor (TNF) was shown to be one of the major secretory products of endotoxin-activated macrophages (29, 30). Passive immunization against TNF caused substantial inhibition of the lethal effect of LPS in vivo (31). In separate studies, TNF was revealed as a strongly proinflammatory mediator, capable of mimicking many of the end effects of endotoxin (32). It would, in fact, provoke shock, coagulation, and widespread tissue injury if administered to laboratory animals. In vitro, it was shown to be capable of activating neutrophils (33), stimulating the release of proteolytic enzymes and prostanoids from diverse cell types (34), and altering endothelial surfaces so as to favor coagulation (35, 36). Although TNF certainly does not work alone and was not the only endogenous mediator of endotoxicity, it seemed that the central sequence of events had been illuminated. LPS triggers the activation of macrophages; macrophages release TNF and other cytokine mediators; these mediators act on many tissues throughout the body to cause inflammation and shock.

This general scheme of events did not address questions as to how LPS might be recognized in the first instance. Separate lines of inquiry began to bring this issue into sharp focus.

THE LPS SIGNALING COMPLEX

In 1990, CD14 was shown to be a biologically relevant receptor for LPS on the surface of mononuclear cells (37). When transfected to express CD14, 70Z3 cells acquired sensitivity to LPS, and antibodies against CD14 blocked the LPS response (38). At the same time, a plasma protein called LBP was revealed as an LPS carrier protein (39), conveying LPS to the surface of cells where it became bound to CD14. CD14 could not, however, transduce a signal across the plasma membrane by itself. Being a GPI (glycosylphosphoinositol)-anchored protein, it lacked a cytoplasmic domain. Hence, a missing coreceptor for LPS was assumed to exist.

There seemed to be some hope of identifying such a receptor from the inside out. TNF, by this time taken as an important endpoint of LPS responses, was synthesized as a result of separate transcriptional and translational activation events. Enhanced TNF gene transcription in myeloid cells followed LPS activation as a result of translocation of NF-κB to the nucleus (40). Translational activation depended upon de-repression of a UA-rich element in the 3′-untranslated region of the TNF mRNA (41). Subsequently, this event required activation of p38 (43), a protein that was first identified because it became phosphorylated in endotoxin-activated macrophages (44). In addition, LPS activated the MAP kinase pathway (45) and PI3 kinase pathway (46–49). The added importance of a tyrosine kinase in LPS signaling was suggested by the fact that tyrosine kinase inhibitors could block signal transduction (50). All attempts to find the critical transmembrane receptor that initiated these events were unsuccessful.

Between 1993 and 1998, the LPS gene, which was defective in C3H/HeJ mice, was positionally cloned by Poltorak et al. (51, 52) and shown to encode the Toll-like receptor 4 (TLR4). Prior to this effort, the Toll-like receptors were known only for their similarity to Toll, a bifunctional plasma membrane protein involved in both development and innate immune responses in *Drosophila melanogaster*. Of critical importance, TLR4 was found to be one member of a family of paralogous proteins in mammals (53–57), now known to include ten members in humans (58–60). The fact that one member of the family, TLR4, was highly specific as a mediator of endotoxin responses suggested that each member of the family might recognize a separate set of microbial products.

This supposition proved to be the case when, in 1999, gene knockout work revealed that TLR2 was required for biological responses to bacterial lipopeptides and peptidoglycan (61). In 2000, TLR9 was shown to be required for responses to unmethylated bacterial DNA (62). Later, TLR5 was shown to carry the flagellin signal (63), whereas TLR3 was associated with signaling initiated by double-stranded RNA (64). Collectively, the Toll-like receptors seem to sense much of the microbial world.

Where the endotoxin receptor is concerned, a third component (at least) seems to be required: a small exteriorized protein known as MD-2. The role of MD-2 in LPS signaling was revealed by transfection studies, in which 293 cells made to express CD14 and TLR4 were refractory to LPS signaling (65). However, coexpression of MD-2 would confer an ability to signal to the level of NF-κB activation. Moreover, MD-2 has been shown to be tightly associated to the ectodomain of TLR4, through analysis of interactions between specific monoclonal antibodies that recognize only the complex of the two proteins (66). Although no other components of the receptor complex are known to exist, it is possible that they do. Because TLR4 exists at a very low concentration on the surface of macrophages (67), tremendous signal amplification must occur to convey the lethal effect of LPS. From a practical standpoint, it is difficult to identify new components of the LPS receptor complex, should they exist, using conventional biochemical methods.

THE EVOLUTION OF TOLL-LIKE RECEPTORS AND THE TIR DOMAIN

Whence Toll? As already mentioned, there are ten TLR paralogs in humans, whereas in flies, a set of nine such receptors exists (58). The namesake of the family, Toll, has an immune function in flies, responding to signals initiated by fungi (68) or gram-positive organisms (69, 70). A proteolytic cascade is triggered in response to these stimuli, leading to the cleavage of pro-spätzle, a prohormone, generating the ligand spätzle, which engages Toll. Toll then signals by way of at least three proteins: tube (a protein of unknown function), MyD88 (a conserved adapter protein with homology to Toll itself), and pelle (a serine kinase) to initiate a signal. The production of an antimicrobial polypeptide, drosomycin, is triggered by translocation of dif (71), an NF-κB homologue, to the nucleus after activation of the upstream signaling components just mentioned.

The discovery that Toll has an antimicrobial function in *Drosophila* was the product of pure genetic work carried out by Hoffmann and colleagues (68), who earlier recognized that the diptericin and drosomycin genes respond to NF-κB-like signals (72–75), and who were aware of the potential for Toll to activate NF-κB. At the time they performed their studies, it had already been shown that in mammals, a protein of immunologic importance also signals by way of a Toll-related receptor (76). The receptor in question was one of two that recognized the inflammatory cytokine IL-1 (77). On its cytoplasmic side, both chains of the Type I IL-1 receptor are now known to be homologous to Toll. IL-1 signals traverse MyD88, IRAK, and NF-κB (78), leading to the activation of many genes involved in the inflammatory response.

The first mammalian Toll-like receptor was cloned in 1994 by Nomura and colleagues (53). In 1996, Taguchi et al. mapped the gene encoding this protein, later known as TLR1, to chromosome 4 in humans (54). Because it was not yet known that Toll had an immunologic function, Taguchi and colleagues did not suspect that the protein was involved in immune responses; rather they suspected that it might play a role in mammalian development (54). The 1996 discovery that Toll protects flies against fungal infection (68) foreshadowed the 1998 discovery that TLR4, one of the mammalian homologues of Toll, played a role in the containment of gram-negative infection (51, 52). Prior to the determination that TLR4 was the mammalian LPS receptor, it was shown that the protein was capable of activating NF-κB translocation to the nucleus of cells, much as Toll and IL-1R had been shown to do (55). However, this finding could not enlighten understanding of function. Were the mammalian TLRs developmentally or immunologically important? Or perhaps both?

It is now believed that the developmental function of the Tolls in flies (so-called to distinguish them from the mammalian TLRs) is something of an evolutionary digression. This belief is predicated largely on the fact that in still more divergent species (notably plants), the conserved TIR domain (Toll/IL-1 receptor/resistance), which comprises most of the cytoplasmic domain of all of the Toll-like receptors,

has a defensive function (79). Therefore, in the fly, it seems most probable that the TIR domain was co-opted to serve a developmental purpose. This type of adaptation has not yet been observed elsewhere in the phylogenetic tree. A hint that such adaptations may be possible comes from the work of Weinmann and colleagues, who observed that TLR4 signaling may cause changes in chromatin structure, assessed by nucleosome placement (80). Hence, it may be that some organisms find it a short leap from NF-κB signaling to genuine developmental change.

All of the Toll-like receptors have a series of leucine-rich repeat motifs scattered throughout the ectodomain region and have a cytoplasmic domain that is composed mostly of a conserved TIR domain. The TIR domain can be used for evolutionary studies by constructing hidden Markov models (81). Moreover, a reasonable calibration standard for divergence can be produced based on the measurement of genetic distance between such orthologs as fish and mammalian TLR3, bird and reptilian TLR2, and bird and mammalian TLR2. On this basis, it has been calculated that TLR4, the endotoxin sensor, diverged from other TLRs near the dawn of vertebrate evolution (82). However, as of this writing, TLR4 has been identified only in mammals. Correspondingly, only mammals are highly susceptible to LPS. Some TLRs are clearly lost from the genome over a relatively short period of time: For example, no TLR10 sequence can be found in the mouse genome as currently represented in the Celera database or in the sequences captured by the public consortium. Only a single TLR, TLR3, is currently represented among *Danio rerio* (zebrafish) sequences. These evolutionary choices may reflect liabilities of certain TLRs that become evident with speciation, as vertebrates adapt to new and different pathogens.

TLR STRUCTURE

It is believed that TLR4 is a homodimer because enforced dimerization of TLR4 creates constitutive signaling activity (55). It is likely that TLR4, MD-2, and CD14 form a fairly tight complex with one another during endotoxin signaling because all can be labeled with photochemically activated lipid A derivatives. Furthermore, fluorescence resonance energy transfer (FRET) analyses suggest that LPS brings CD14 into close contact with the TLR4 MD-2 complex (83).

Though Toll does not come into direct contact with any product of fungi, TLR4 does have direct contact with LPS. This has been demonstrated through genetic techniques. Whereas human cells are induced to make TNF in response to lipid A but not tetra-acyl lipid A, mouse mononuclear cells respond to both stimuli (84). Transfection studies have revealed that the species-dependent difference in response is solely attributable to structural difference between human and mouse TLR4 (85, 86). In this sense, human TLR4 is able to make a distinction as to whether secondary acyl chains are present on the agonist molecule. To do so, it must be in very close proximity to the agonist, which is to say that it most likely engages in direct physical contact with it. The nature of the complex that is formed and the conformational changes that occur following engagement of LPS is a

subject of great interest and will probably only be resolved by crystallographic studies.

Crystallography has already shown the basic protein fold of the TIR domain, and it is interesting that modification of the TIR domain of TLR2 by "engrafting" the *Lps*^{d} mutation of the C3H/HeJ mouse does not change its tertiary structure in a major way (87). The modified protein is still crystallizable and retains its overall fold. Based on in vitro mutagenesis studies (67), it seems likely that the modification imposed by the *Lps*^{d} mutation leads to a change in the association between subunits of the molecule. Deleting the entire TIR domain does not have a codominant effect on LPS signaling as does the *Lps*^{d} mutation (67). Although it might be argued that the *Lps*^{d} mutation sequesters a downstream signaling molecule like MyD88, this seems unlikely because the mutation does not suppress signals through any of the other Toll-like receptors, as would happen if MyD88 were bound up in an association with TLR4.

SIGNAL TRANSDUCTION FROM THE ENDOTOXIN RECEPTOR

As with the activation of IL-1 (78), the activation of TLR4 leads to recruitment of MyD88 (88), a TIR domain–bearing protein that also has N-terminal death domains. By death domain interaction, MyD88 forms a complex with IRAK, or IRAK4, both of which are capable of transducing the LPS signaling. Knockout work suggests that IRAK4 is of primary importance (89). Mice lacking IRAK4 show almost complete insensitivity to LPS. IRAK (and presumably IRAK4) activates TRAF6 (90), which in turn activates NIK (90) and TAK1, the latter in a process that depends upon TAB1 and TAB2 (91). TAB1 may also signal toward activation of MAPK, permitting activation of TNF mRNA translation (92). TAK1 phosphorylates signalosome proteins, which in turn phosphorylate IκB, which permits NF-κB translocation to the nucleus.

Recently, MAL (93) [Tirap (94)] has been identified as a TIR domain-containing protein that also seems to participate in LPS signaling. MAL/Tirap also engages the TLR4 receptor and signals the activation of MAP kinase, p38, and NF-κB. The relative contribution of MAL/Tirap and MyD88 to activation can be properly assessed only with the knockout of MAL/Tirap. It is known that MyD88 is important in endotoxin signaling because targeted deletion of the MyD88 gene creates strong insensitivity to LPS (95).

Other signaling proteins may exist. In humans, a mutation is known to abolish sensitivity to LPS and MyD88. However, this mutation has not been traced to either the MyD88 gene, the IRAK4 gene, or to any other known component of the LPS-specific signaling pathway (95a).

Similarities between TLR signals seem to exceed differences. Although selected endpoints of signaling do show specificity with regard to the receptors that initiate them (96), many of the cytokines that transduce the LPS effect are shared in

common with those that transduce the lipopeptide effect or the effects of unmethylated DNA. TNF provides a ready example: Its synthesis is induced by TLRs 2, 4, and 9. Moreover, certain phenomena that have long been studied in the LPS field seem to apply to ligands that transduce their effects through TLRs other than TLR4.

One such phenomenon is endotoxin tolerance. It is known that LPS stimulation is associated with a prompt response (NF-κB translocation to the nucleus and cytokine production) followed by a refractory state, wherein a second challenge is far less effective at provoking such a response (97, 98). Cross-tolerance has been observed when a primary stimulus with lipopeptides is used in place of LPS (99). Although some have attributed tolerance to the production of antiinflammatory cytokines such as TGFβ and/or IL-10 (100), it is more widely held that tolerance reflects the activation of a feedback pathway within cells, causing paralysis of the LPS response. One example of tolerance at the cellular level involves the production of NF-κB p50 homodimers, which can bind to diverse promoters within the cell and prevent activation by p50/p65 heterodimers (101). Other levels of blockade are also possible and are currently under investigation.

A number of agents sensitize to endotoxin. Some are hepatotoxic agents, including lead acetate and D-galactosamine, that seem to sensitize by encouraging TNF-mediated apoptosis of cells in the liver (102). On the other hand, cytokines including interferon-γ sensitize to LPS by lowering the activation threshold of the macrophage population and increasing the amount of TNF that is produced in response to a given LPS challenge (103, 104). Agents of the latter class are more interesting in the sense that their mechanism of action within macrophages remains to be discovered. It is possible that they hold some clinical relevance insofar as priming states [the infection of mice with *Bacillus Calmette-Guerin* (BCG) or *P. acnes*] seem to depend on the production of endogenous cytokine mediators. When primed in this manner, mice may be 10,000-fold more sensitive to challenge with LPS than normal animals (105).

THE EFFECT OF MUTATIONS AT THE TLR4 LOCUS

TLR4 has been very heavily sequenced in an effort to determine how polymorphic it might be. Analyses of synonymous and nonsynonymous substitution within the TLR4 coding region have led to the conclusion that the gene is subject to weak purifying selection (106). That is, it seems to resist structural change and does not undergo promiscuous modification along the lines of some immune proteins that have direct contact with the microbial world (e.g., the MHC antigens). Most changes in the TLR4 coding sequence are weakly deleterious. For this reason, mutational changes have not risen to a high frequency in human populations. Among Caucasians, a double amino acid substitution within the midectodomain of the TLR4 molecule has been observed. This mutation seems to diminish responses to LPS in vivo (107). However, it has not yet been found at high frequency in any human disease.

Rare mutations of TLR4 are observed at higher frequency among patients with severe gram-negative infection (meningococcal sepsis). Such mutations are probably of etiologic importance, and their higher frequency among patients with meningococcal disease cannot be ascribed to linkage disequilibrium with another locus that is of authentic importance (I. Smirnova, N. Mann, M. Hibberd, M. Levin, B. Beutler, manuscript in preparation). The TLRs probably should be regarded as potential susceptibility loci in most infectious diseases, but it is likely that each locus makes only a small contribution to susceptibility for most pathogens.

EVOLUTIONARY CALCULATIONS: THE "SET POINT" OF LPS RESPONSES

As has been emphasized in this review, LPS is an unusual poison in that its mechanism of toxicity has been preserved by evolution. In effect, the host "realizes" that LPS is toxic and accepts the risk of toxicity for the greater good of combating infection. A single mutation would suffice to remove the threat of LPS toxicity, and many species seem to have chosen this option. In mammals, however, LPS sensing is acute, and with this faculty has come the burden of LPS toxicity. What is the nature of the tradeoff in mathematical terms? Can it be calculated?

It must be assumed that the LPS sensing mechanism of mammals was retained to detect small inocula (which might be overcome by innate immune defenses) rather than inocula that are large enough to trigger an injurious or lethal response through the same system. Sensitivity to LPS sets the vigor of the protective response to a small inoculum. But it also limits the microbial burden that can be tolerated without lethality. LPS tolerance, as a general phenomenon, may be viewed as an attempt to buffer the latter effect and extend the flexibility with which the organism may cope with infection.

For a given gram-negative organism (or for all gram-negative organisms that the host will ever encounter) and a given host species—for any and all routes of inoculation—several mathematical relationships might be considered. First, the lethal effect of an inoculum is related to inoculum size (Figure 1*A*), and the mean lethal inoculum (MLI) will generally be found at the point of maximum slope. A given host species will exhibit an acute survivable sensitivity to LPS, indicative of the maximum amount of LPS that can be tolerated. If acute survivable LPS sensitivity is low, then a substantial inoculum might be tolerated acutely. If acute survivable LPS sensitivity is enormous, then even one microbe might kill the host acutely (Figure 1*B*). The acute survivable LPS sensitivity can be estimated for a given species by determining the lethality curve for LPS in vivo. At the same time, the probability of receiving an inoculum per unit time is related to inoculum size as well: Small inocula are much more common than large inocula (Figure 1*C*), but it is also improbable that an organism will sustain no inocula with

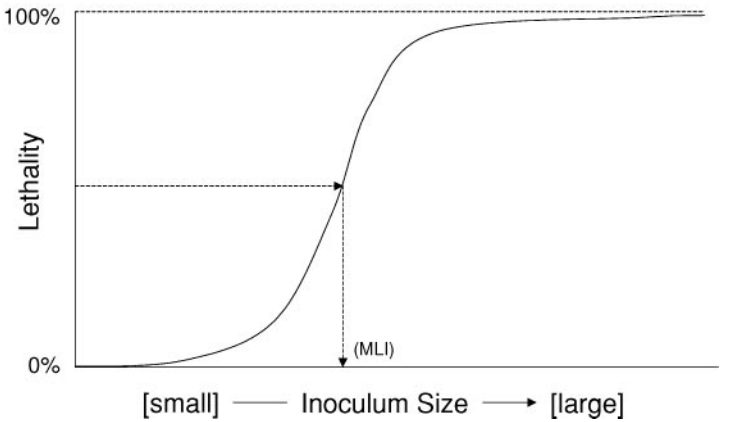

Figure 1*A* The relationship between inoculum size and lethal effect. A sigmoid curve can be expected in experimental analyses and most likely applies under all circumstances.

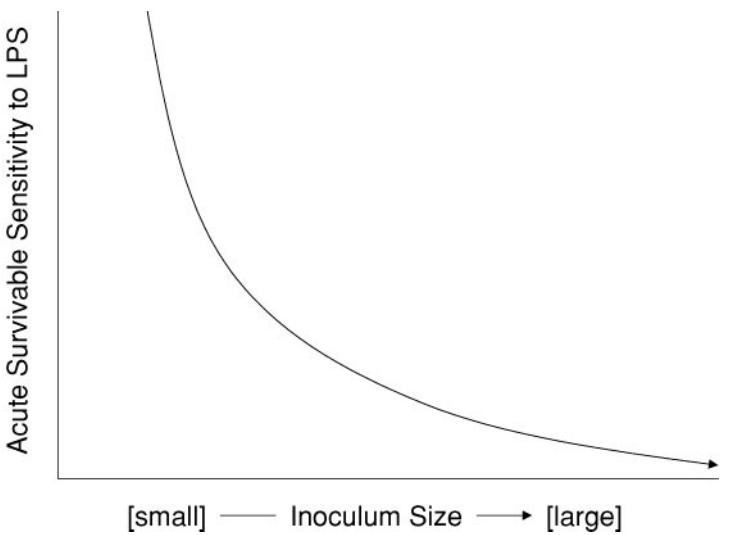

Figure 1*B* The relationship between inoculum size and acute survivable sensitivity to LPS. For any mammalian species, a lethal effect will attend the inoculation of gram-negative organisms, given that the inoculum is large enough. The less sensitive the host is to LPS, the larger the lethal inoculum will be. If sensitivity to LPS is exquisite, even minute inocula may prove lethal acutely.

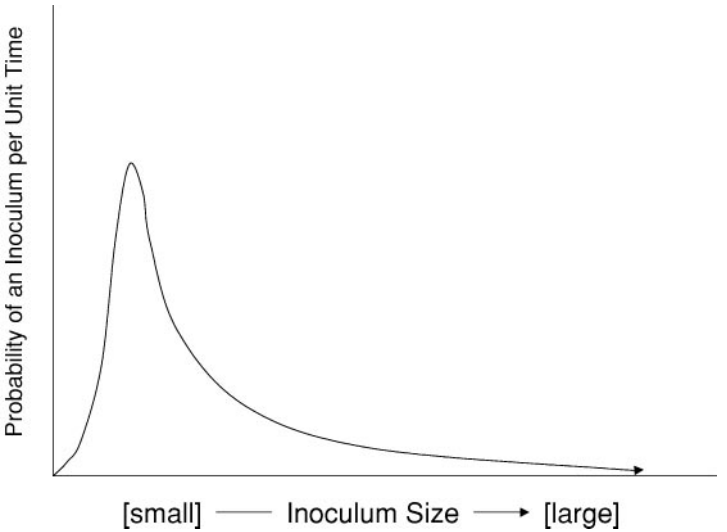

Figure 1*C* The relationship between the likelihood that inoculation will occur within a given length of time and the size of that inoculum. All individuals are exposed to small inocula very frequently, and it is therefore improbable that no inocula will occur per unit time. It is also improbable, however, that very large inocula will occur. Hence, the curve traverses a maximum.

LPS-bearing organisms. The probability of sustaining an infection of a given inoculum size undoubtedly influences the survivable sensitivity to LPS at the same inoculum size because highly probable events must not be lethal events. Therefore, species that are exposed to high concentrations of LPS in the course of life must not have exquisite sensitivity to LPS. In the end, survival at a given inoculum size (for example, at the mean lethal inoculum) can be expressed as a function of LPS-sensing competence: Extremely low LPS sensitivity leads to overwhelming infection; extremely high sensitivity leads to acute death (Figure 1*D*; also see Figure 2).

Given sufficient time, mutation and selection undoubtedly calculate the optimum response of a species to all microbial inducers. However, the relatively rapid shifts that are known to occur in the microbial world may cause a departure from equilibrium. Where our own species is concerned, we cannot conclude that we live in "the best of all possible worlds." An epidemic may alter realities within the space of a few days, confronting the host with microbial challenges that are beyond the established routine, in terms of the route of inoculation, LPS toxicity, and the efficacy of the LPS response. For this reason, it is not clear how one ought to proceed in moderating the LPS response during infection, assuming that one has the means to do so. And certainly, what applies for one infectious agent at one particular moment may not apply universally.

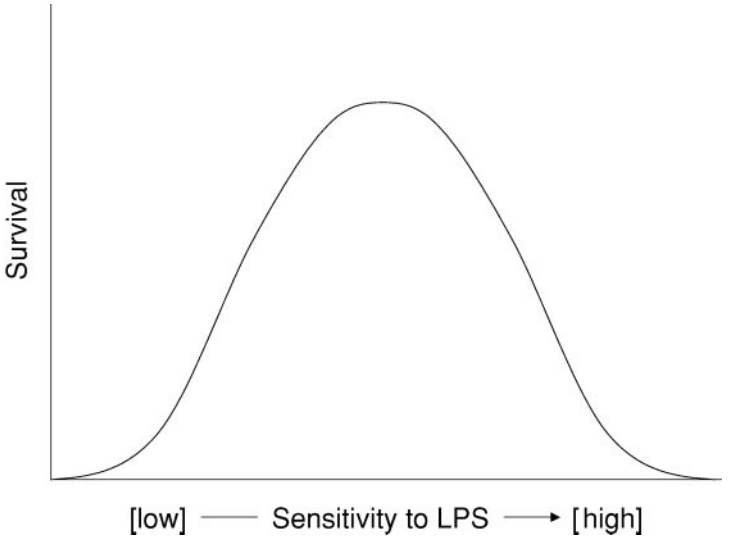

Figure 1*D* Survival as a function of LPS sensitivity. Animals that cannot perceive LPS are at high risk for mortality following gram-negative inoculation. In principle, animals that respond too vigorously to LPS would also be at risk.

PHARMACOLOGIC BLOCKADE OF TLR4 SIGNALING: IS IT POSSIBLE AND WOULD IT BE WISE?

Before our present understanding of LPS signaling was attained, many attempts to interdict the LPS signal were made nonetheless. The first such attempts involved antibodies against LPS, an approach that may be traced to the beginning of the LPS story itself and to the pioneering work of Besredka (1, 2). But attempts to mitigate the toxicity of infections with anti-LPS antibody, though widely publicized (108), have in no instance been widely accepted as successful. There have also been attempts to interdict the signal at the level of CD14—an approach that is still in progress. Anti-TNF antibodies have not shown a beneficial effect during sepsis, despite their ability to protect against LPS in animals (31), perhaps because intervention is too late.

The fact that TLR4, MD-2, CD14, MyD88, and IRAK4 are all critical LPS signaling proteins suggests that it should be possible to fashion small molecular antagonists that will impede signaling, perhaps sparing the host needless injury once an infection has been identified. The belief that antagonists might be produced artificially was encouraged by the fact that tetra-acyl lipid A (84, 109) and certain natural lipid A molecules, like the lipid A of *Rhodopseudomonas sphaeroides* (110), are indeed capable of blocking signal transduction from toxic LPS species. Indeed, it has been possible to create small molecular antagonists that block TLR4 signaling. Among these, E5531 has been tested most extensively (111). It remains

to be seen whether it will be clinically effective, though it does block LPS responses in humans in vivo (112).

Global blockade of TLR signaling might also be achieved, particularly at the level of MyD88 or IRAK4. The latter molecule is a particularly appealing target because it is an enzyme and is probably responsible for the bulk of signal amplification that occurs during LPS activation. Would this be a good idea? Possibly so, though at some point it must be recognized that immune paralysis is detrimental to the host. Even if high doses of antibiotics are administered, sterilizing immunity requires the integrity of myeloid cells, particularly neutrophils, which may, like their mononuclear relatives, rely upon TLRs for detection of pathogens.

SUMMARY AND FUTURE HURDLES

The nature of the LPS sensor was revealed by a spontaneous mutation, and as it happened, the long-cherished belief that LPS was an excellent model for infectious processes turned out to be correct. The LPS sensor was but one member of a paralogous family. When it was revealed, reverse genetic tools (chiefly gene targeting) were swiftly applied to determine the precise function of the other paralogs in the family. Some of the functions of these paralogs have now been deciphered. But certain facts must be borne in mind. Among the remaining TLRs, genuine microbial ligands have yet to be identified for TLRs 1, 6, 7, 8, and 10. TLR10 is not represented in mice and cannot be approached by means of a knockout. And for the others, there will be many phenotypes to test once knockouts are made. Most important, many of the essential molecular participants in signaling may remain to be identified.

The TLRs have occupied center stage for a time, but they attracted notice only because of pioneering forward genetic work—genetic work that begins with phenotype. The key events were the discovery of the immune function of Toll in *Drosophila* and the positional cloning of a spontaneous mutation first observed in mice 37 years ago. There may yet be a long way to go. The essential function of most mammalian genes remains undiscovered, the full complement of genes that subserve most complex functions are mostly undiscovered, and there is no reason to think that innate immune sensing pathways are particularly privileged in this regard.

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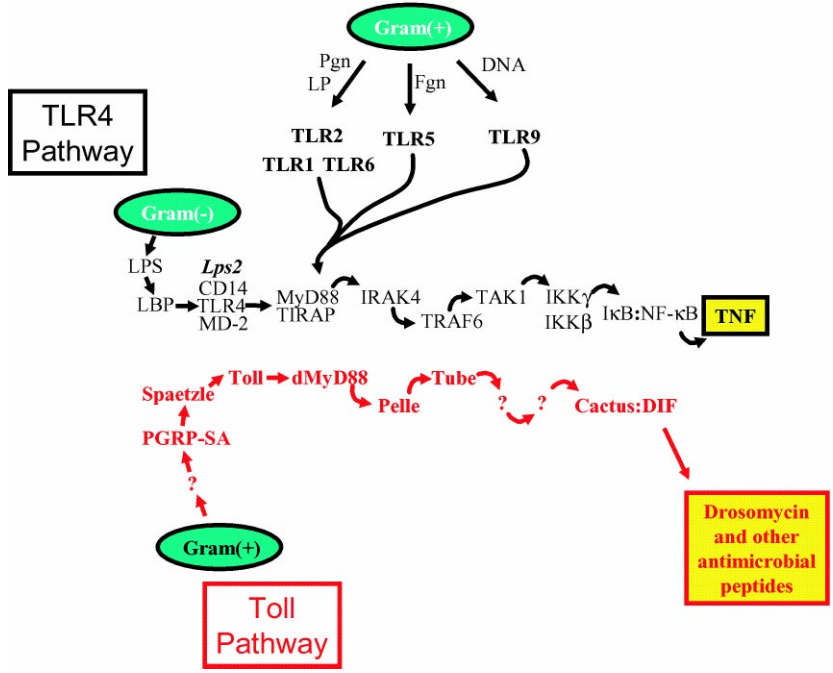

Figure 2 Core elements of the LPS signaling pathway. LPS, released from Gram-negative bacteria, triggers a sequence of events (black) that are largely mirrored by an ancestrally conserved pathway in *Drosophila* (red). At the apex of the LPS signaling apparatus is TLR4, which perceives LPS presented by CD14, and does so in conjunction with MD-2. *Lps2*, a still-unidentified protein known to exist on the basis of forward genetic studies, may also participate in the initial events of signaling. In *Drosophila*, Gram-positive bacteria and fungi are sensed via Toll, but there is no evidence of direct interaction between any molecule of microbial origin and the Toll protein itself. TLRs 1, 2, and 6 are involved in sensing peptidoglycan (Pgn), lipopeptides (LP), and probably other microbial molecules; TLR5 senses flagellin (Fgn); and TLR9 senses microbial DNA. All of these molecules feed signals into the core-sensing pathway at the level of MyD88 interaction. TNF production is probably the single most important terminal event where LPS toxicity is concerned, and all activators of TLR proteins elicit this response to one degree or another.